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Exosome-Mediated Crosstalk between Keratinocytes and Macrophages

in Cutaneous Wound Healing

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ABSTRACT: Bidirectional cell-cell communication involving exosome-borne cargo such as miRNA, has emerged as a critical mechanism for wound healing. Unlike other shedding vesicles, exosomes selectively package miRNA by SUMOylation of heterogeneous nuclear ribonucleoproteinA2B1 (hnRNPA2B1). In this work, we elucidate the significance of exosome in keratinocyte-macrophage crosstalk following injury. Keratinocyte-derived exosomes were genetically labeled with GFP reporter (Exo_{K-GFP}) using tissue nanotransfection and were isolated from dorsal murine skin and wound-edge tissue by affinity selection using magnetic beads. Surface N-glycans of Exo_{K-GFP} were also characterized. Unlike skin exosome, wound-edge Exo_{K-GFP} demonstrated characteristic N-glycan ions with abundance of low base pair RNA and were selectively engulfed by wound-macrophages ($\omega\phi$) in granulation tissue. *In vitro* addition of wound-edge Exo_{K-GFP} to proinflammatory $\omega\phi$ resulted in conversion to a proresolution phenotype. To selectively inhibit miRNA packaging within Exo_{K-GFP} *in vivo*, pH-responsive keratinocyte-targeted siRNA-hnRNPA2B1 functionalized lipid nanoparticles (TLNP_K) were designed with 94.3% encapsulation efficiency. Application of TLNP_{K/si-hnRNPA2B1} to murine dorsal wound-edge significantly inhibited expression of hnRNPA2B1 by 80% in epidermis compared to TLNP_{K/si-control} group. Although no significant difference in wound closure or re-epithelialization was observed, TLNP_{K/si-hnRNPA2B1} treated group showed significant increase in $\omega\phi$ displaying proinflammatory markers in the granulation tissue at day 10 post-wounding compared to TLNP_{K/si-control} group. Furthermore, TLNP_{K/si-hnRNPA2B1} treated mice showed impaired barrier function with diminished expression of epithelial junctional proteins, lending credence to the notion that unresolved inflammation results in leaky skin. This work provides insight wherein Exo_{K-GFP} are recognized as a major contributor that regulates macrophage trafficking and epithelial barrier properties post-injury.

KEY WORDS: exosome, tissue nanotransfection, keratinocyte-targeted lipid nanoparticles, macrophage, wound healing.

The dynamic cellular events following cutaneous injury rely on bidirectional cell-cell communication for efficient wound healing. Such crosstalk is traditionally known to occur *via* paracrine effects.^{1, 2} A recent paradigm has emerged wherein the predominant mechanism of cellular communication is attributable to extracellular vesicles (EV).³⁻⁷ These EV have distinct structural and biochemical properties depending on their intracellular site of origin that affects their biological function.⁸ A majority of these vesicles having diameters ranging from 50-1000 nm originate from the plasma membrane and are often referred to as microvesicles, ectosomes, microparticles, and exovesicles.⁸ Exosomes are a major class of EV (typically 30–150 nm in diameter) of endocytic origin released by all cell types following fusion of multi-vesicular bodies (MVB) with the plasma membrane.^{9, 10} Exosomes carry a distinctive repertoire of cargo such as miRNAs.¹¹ RNA profiling of exosomes showed differences in miRNA abundance compared to the parent cells, suggesting that parent cells possess a sorting mechanism that guides specific intracellular miRNAs to enter into the exosomes.¹²⁻¹⁴ SUMOylation of heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) has been implicated as a predominant mechanism of miRNA packaging within exosomes.¹⁵ Within the cell, miRNAs have specific sequence motifs that control their localization into exosomes. Binding of hnRNPA2B1 to the miRNA by recognition of these motifs controls their loading into exosomes. Moreover, SUMOylation of hnRNPA2B1 regulates the binding of hnRNPA2B1 to miRNAs. Given the presence of such a well-coordinated sorting mechanism, it is apparent that cell-cell crosstalk *via* exosomes is an active process. Such process is distinct from cellular communication mediated *via* shedding of other membrane vesicles that also carry biomolecules such as miRNA as cargo that is not selectively packaged in these vesicles. Lack of efficient isolation techniques of exosomes from other membrane vesicles of similar size have led to conclusions that are primarily derived from a heterogenous EV pool. This lack of discrimination among various membrane vesicles dampens

the potential significance of exosomes in cellular communication and has impaired discovery of the role of exosome of specific cellular origins in communication.

The putative role of keratinocytes in wound healing and inflammation is well documented.¹⁶⁻¹⁹ At the wound site, cells of myeloid origin such as monocytes and macrophages are primarily responsible for mounting an early inflammatory response to injury.²⁰⁻²³ Both robust mounting of inflammation as well as timely resolution are key to successful tissue repair. The role of keratinocytes for the resolution of inflammation remains unclear and debated. This study rests on our observation that at the site of injury, EV of keratinocyte origin are critical for conversion of the myeloid cells into fibroblast-like cells in the granulation tissue.¹⁹ The objectives of this work were to isolate exosomes of keratinocyte origin at the wound-edge and to delineate their significance in the resolution of inflammation at the wound site. This work shows that keratinocyte derived exosomes carry miRNAs that direct resolution of macrophage numbers and function within the granulation tissue and are critical for functional wound closure.

RESULTS AND DISCUSSION

Identification and isolation of keratinocyte-derived exosomes in murine skin. To circumvent the EV complexity found *in vivo* in complex tissues, we employed an *in vitro* system and isolated exosomes from the heterogenous pool of EV derived from cultured keratinocytes. According to EVPedia²⁴ and Exocarta,²⁵ the three tetraspanins CD9, CD63 and CD81 are reliable markers of exosomes.²⁶ In the interest of rigor, we employed a two-step process to isolate pure exosome. First, EV were isolated from keratinocyte-conditioned media using differential ultracentrifugation (Figure S1A). Second, the heterogenous EV were incubated with superparamagnetic Dynabeads™ conjugated with antibodies for CD9, CD63 and CD81 (Figure S1A). From the heterogenous EV mixture, only the tetraspanins expressing exosomes were trapped leaving the membrane particles

1 and apoptotic bodies in the flow through. We tested all the nine criteria set forward by EV-track
2 for transparent reporting (Figure S1). The size and concentration of the exosomes were analyzed
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4 by nanoparticle tracking analysis (NanoSight™) and Scanning electron microscopy (SEM) (Figure
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6 S1B-C). The density of the isolated exosomes was found to be 1.16g/ml. The isolated exosomes
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8 showed abundance of other reported exosome markers such as Alix, TSG101, HSP90 and Flotilin
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10 1 (Figure S1D). The purity of the isolation was tested by immunoblotting of GM130 and Prohibitin
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12 that are reported as major contaminants of exosome preparation by EV-track.²⁷ Additional
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14 quantitative analysis such as flow cytometry of these Dynabeads™ post-adsorption with exosomes
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16 following incubation with phycoerythrin (PE) conjugated TSG101 (TSG101-PE) antibody showed
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18 an increased shift in fluorescence intensity (Figure S1E). Furthermore, binding of PE tagged
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20 TSG101 antibody was tested by fluorescence anisotropy (Figure S1F) and fluorescence correlation
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22 spectroscopy (Figure S1G). Binding of TSG101-PE by the resuspended isolated exosomes
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24 hindered their free rotation as shown by a marked increase in fluorescence anisotropy (Figure S1F).
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26 TSG101-PE had fast and unhindered diffusion when resuspended in PBS or in the presence of EV
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28 flow through. However, in the presence of exosomes, diffusion of TSG101-PE slowed down as
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30 shown by shifted autocorrelation curve (Figure S1G). These data validated that the two-step
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32 isolation process was successful in separating exosomes from the heterogenous EV pool. This
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34 method of exosome isolation was reported in EV-track (EV190103) with a preliminary EV-
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36 METRIC of 100%.²⁸
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48 The approach described above was applied for isolation of exosomes *in vivo*. Recent reports have
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50 highlighted the role of exosome in cutaneous wound healing.^{29, 30} Analysis of EV concentration
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52 from murine skin and day 5 wound-edge tissue (< 2mm from wound-edge) showed significant
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54 increase in EV as well as exosomes in day 5 wound-edge tissue (Figure 1A). Quantitative analysis
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56 revealed that exosome represents only about 10% of the total EV pools. In addition to the resident
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1 cells such as keratinocytes and fibroblasts, day 5 wound-edge tissue contains infiltrated blood-
2 borne cells including macrophages. Thus, to understand whether the increase abundance of
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cells such as keratinocytes and fibroblasts, day 5 wound-edge tissue contains infiltrated blood-borne cells including macrophages. Thus, to understand whether the increase abundance of exosome at the wound-edge is due to increased release of exosomes by additional biogenesis in resident cells or contributed by infiltrating cells, it becomes critical to segregate the exosomes based on their cellular origin.

Our previous work has recognized the critical role of keratinocytes derived EV in the conversion of injury-site myeloid cells to fibroblast-like cells in granulation tissue.¹⁹ However the significance of exosomes in such keratinocyte-myeloid cell crosstalk remains unknown. Since one or more of the three tetraspanins CD9, CD63 and CD81 are expressed in all exosomes, we designed three murine keratin 14 (K14) promoter-driven plasmids that encode for CD9, CD63 and CD81 with “in frame” GFP reporter (Figure 1B). Such promoter-driven plasmids allow expression of GFP tagged CD markers only in keratinocytes. The specificity of the plasmid cocktail was tested in murine keratinocytes, fibroblasts and $\omega m\phi$ (Figure S2A-C). We tested the hypothesis that *in vivo* topical delivery of these three plasmids would result in the expression of GFP in all exosomes that are of keratinocyte origin. We have previously reported that cutaneous delivery of reprogramming molecules *via* tissue nanotransfection (TNT) was efficient and effective in directly reprogramming dermal fibroblast cells into a variety of functionally distinct lineages.³¹ We have now generated a modified TNT silicon chip (TNT2.0) with longer needle height of 170 μ m and pore diameter of 4 μ m (Figure 1C). Modifying the electrical potential applied between the cargo within the chip and skin led to stepwise increases in the depth of transfection of fluorescein amidite (FAM)-labeled DNA (Figure 1D, Figure S2D) in wild type C57BL/6 mice. Delivery of these three K14 promoter-driven plasmids *via* TNT followed by super-resolution confocal microscopy demonstrated presence of exosome with GFP reporter expression in epidermis as well as in dermis

(Figure 1E, Figure S2E). Because the GFP reporter protein was cloned in frame with the CD9, CD63 and CD81, pull down of GFP using magnetic traps is expected to isolate the $\text{Exo}_{\text{K-GFP}}$. Keratinocyte-derived exosomes were isolated from the tissue homogenate using GFP-magnetic traps followed by separating the exosomes from the beads using elution buffer (Figure 1F). The resultant flow through was further ultracentrifuged and the non-keratinocyte derived exosome ($\text{Exo}_{\text{non-GFP}}$) were isolated using pan-CD superparamagnetic beads (Figure 1F). The size and concentration of $\text{Exo}_{\text{K-GFP}}$, $\text{Exo}_{\text{non-GFP}}$ and flow through were further examined using nanoparticle tracking analysis (Nanosight™) (Figure 1G). Scanning electron microscopy of $\text{Exo}_{\text{K-GFP}}$ revealed presence of exosomes of different size (Figure 1H). Adsorption of $\text{Exo}_{\text{K-GFP}}$ on GFP-Trap was tested by fluorescence correlation spectroscopy (Figure 1I) and fluorescence anisotropy (Figure 1J). Flow cytometric analysis of GFP-trap beads post-adsorption with $\text{Exo}_{\text{K-GFP}}$ following incubation with fluorescently tagged antibodies of exosome marker such as TSG101-PE, Alix-FITC, Flotilin1-PE and HSP90-FITC showed an increase in fluorescence intensity (Figure 1K). These findings demonstrate that the GFP-trap approach was successful in isolating $\text{Exo}_{\text{K-GFP}}$ from murine tissue homogenate.

Ultracentrifugation is widely held as a gold standard for exosome isolation. In the interest of rigor, $\text{Exo}_{\text{K-GFP}}$ was subjected to direct comparison with exosomes isolated using differential ultracentrifugation. Murine keratinocytes were transfected with the three K14 promoter-driven plasmids to label the exosomes with GFP-reporter. Exosomes were isolated from the cell culture supernatant using GFP-Trap and compared with exosomes isolated using ultracentrifugation (Figure 2A). No difference in size, shape and binding property were observed between the exosomes isolated using the two methodologies (Figure 2B-F).

Characterization of keratinocyte-derived exosomes isolated from murine skin and wound-edge tissue. Isolation of Exo_{K-GFP} from wild type mice showed a significant increase in day 5 wound-edge Exo_{K-GFP} compared to skin (Figure 3A). High-resolution automated electrophoresis of RNA isolated from Exo_{K-GFP} showed abundance of small bp RNA (<100 bp) in day 5 wound-edge tissue (Figure 3B). These data suggest Exo_K-borne miRNA signals are dispatched from resident keratinocytes of the wound-edge tissue to enable crosstalk with visiting immune cells.

N-glycans molecule facilitate the uptake of keratinocyte-derived exosomes by macrophage at the wound-edge. Based on our previous work that demonstrated the critical role of EVs of keratinocyte origin in the conversion of injury-site myeloid cells to fibroblast-like cells of granulation tissue,¹⁹ it is plausible that the exosomes released from the keratinocytes are likely engulfed by the $\omega\phi$. Immunohistochemistry followed by super-resolution confocal microscopic images revealed that unlike the skin adjacent to wound, the Exo_{K-GFP} at the wound-edge were selectively engulfed by the $\omega\phi$ (Figure S3A-C). Macrophage has abundant membrane-bound C-type lectin receptors that share a common carbohydrate recognition domain.³² Such receptors are critical for binding to specific carbohydrate structures of endogenous self-molecules.³³ The C-type lectin receptors on a macrophage membrane are able to bind branched sugars with terminal mannose, fucose, or N-acetyl-glucosamine, and mediate both endocytosis and phagocytosis.³⁴ Intrigued by the site specific selective uptake of Exo_{K-GFP} by the $\omega\phi$, we tested the hypothesis that at the wound-edge, the Exo_{K-GFP} surface is selectively modified to facilitate uptake by the immune cells. Exo_{K-GFP} N-glycans were analyzed by capillary electrophoresis-mass spectrometry (CE-MS) to identify N-glycan compositions.^{35, 36} Released, purified, and methylamidated N-glycans were labeled with Girard's reagent T (GT) to impart a single positive charge for electrophoretic separation and enhanced ionization efficiency during CE-MS analysis. The

compositions and tentative structures of the glycans were based on detected mass and ExPASy GlyConnect database.³⁷ Through CE-MS, 51 N-glycan compositions were identified in total (Table S1), among which 19 and 7 glycans were exclusively present in skin and day 5 wound-edge tissue, respectively. Furthermore, N-glycans conserved in both skin and day 5 wound-edge differ in their relative intensities (Figure 4). Glycans with m/z 855.349 (corresponding to Hex₄HexNAc₃Neu5Gc₁) existed in both Exo_{k-GFP} samples with similar relative intensities. However, glycan ions at m/z 877.8542 (Hex₅HexNAc₄) were more abundant in skin Exo_{k-GFP}, whereas glycan ions at m/z 707.281 (Hex₆HexNAc₅) were found exclusively in skin Exo_{k-GFP}. Similarly, glycan ions at m/z 720.232 (Hex₁₀HexNAc₂) were only detected in wound edge Exo_{k-GFP}. The difference in abundance of glycan molecules on Exo_{k-GFP} surface lends credence to the notion that they play a role in uptake by $\omega\phi$ during wound healing. *In vitro* inhibition of the C-type lectin receptors in day 3 $\omega\phi$ resulted in decreased uptake of EXO_{k-GFP} (Figure S3C-D and Supp. Movie S3-4). It was thus of interest to test whether Exo_{k-GFP} determines the fate of myeloid cells at the wound site. The conventional M1/M2 nomenclature is not appropriate for $\omega\phi$.^{23, 38, 39} Recognizing the ambiguity in macrophage nomenclature specifically for tissue macrophages,⁴⁰⁻⁴² we classify *in vivo* $\omega\phi$ on the basis of the proinflammatory or proresolution/healing phenotype. Incubation of day 3 $\omega\phi$ (proinflammatory phenotype) with Exo_{k-GFP} and Exo_{non-GFP} isolated from day 3 wound-edge tissue (Figure S3E) caused overt phenotypic changes. On day 7, down-regulation of proinflammatory genes such as *Tnfa*, *Nos2*, *Cd74* was associated with upregulation of the proresolution gene *Cl3* (Figure S3F).

Design and synthesis of keratinocyte specific lipid nanoparticles to inhibit miRNA packaging within exosome. Exosomes carry a distinctive repertoire of cargo such as miRNAs.¹¹ Previous studies on RNA profiling of exosomes showed difference in miRNA abundance compared to the

parent cells, suggesting that parent cells possess a sorting mechanism that guides specific intracellular miRNAs to enter into the exosomes.¹²⁻¹⁴ Should the cues for conversion of wound macrophage from a proinflammatory to a proresolution state be driven by keratinocytes *via* miRNA packaged in exosome, then inhibition of such packaging machinery would result in accumulation of proinflammatory macrophage *in vivo*. SUMOylation of heterogeneous nuclear ribonucleoprotein (hnRNPA2B1) has been implicated in miRNA packaging within exosomes.¹⁵ ExomiRs contain a distinct signature motif known as exomotif. Exomotif is recognized by heterogeneous nuclear ribonucleoprotein (hnRNPA2B1). Exosomal loading of exomiRs is thus controlled by hnRNPA2B1.¹⁵ SUMOylation of hnRNPA2B1 causes gain in function, thus improving exosomal loading of miRNA. Thus, it is plausible that inhibition of hnRNPA2B1 in the keratinocytes would impair the microRNA packaging in Exo_{K-GFP}. We have previously reported keratinocytes targeted delivery of oligonucleotide using functionalized lipid nanoparticles.⁴³ Using a similar approach, we designed keratinocyte-targeted siRNA functionalized lipid nanoparticles (TLNP_K) to inhibit the expression of hnRNPA2B1 in keratinocytes (Figure 5A). Unlike our previous report, the DODAP was replaced by DODMA containing pH-sensitive tertiary amine (Figure 5A). Additionally, the keratinocyte-targeting peptide ASKAIQVFLLAG (A5G33) peptide was conjugated with NHS-PEG₂₀₀₀-DSPE in one-step process for better purification (Figure 5B). A high concentration of Tween 80 (20 mol%) used in nanoparticle formulation for a reduction in the particle size, stabilization of the nanoparticle and increased the skin permeability for local delivery. The Zeta potentials (ζ) of TLNP_K changed from +20 mV to -20 mV in the pH range of 3 to 10 demonstrating the pH-sensitive activity of TLNP_K (Figure 5C). Within physiological limit, the neutral or mild positive charge of the lipid nanoparticles evades uptake by the reticuloendothelial system (RES), prolong circulation time and reduce the toxicity.⁴⁴⁻⁴⁶ The siRNA encapsulation efficiency was found to be more than 90% when measured by Quant-iT RiboGreen

RNA Kit . Gel retardation assay further demonstrated that the siRNA formed complexes with lipid nanoparticles (Figure 5D). The average size of the lipid nanoparticles was found to be 108.9 nm (Figure 5E-F). In a mixed culture comprising of keratinocytes, endothelial cells, and fibroblasts, TLNP_κ was specifically taken up by the keratinocytes within 4 hours demonstrating targeting specificity and efficiency (Figure 6A-B, Figure S4A-B). This TLNP_κ was not cytotoxic in keratinocytes as determined by LDH release (Figure 6C), metabolic viability (Figure S4C), and propidium iodide exclusion (Figure S4D). The uptake of TLNP_κ by murine keratinocytes was more rapid compared to non-TLNP_κ (Figure 6C, Figure S4E). All materials used for its formulation have prior history of FDA approval for human use and thus offer a clear translational advantage.

Functional characterization of keratinocyte-specific lipid nanoparticles to inhibit miRNA packaging within exosome. To test whether our siRNA functionalized keratinocyte-targeted lipid nanoparticles are effective in inhibiting the packaging of the miRNA within Exo_κ-GFP, we utilized commercially available XMIR technology from SBI System Biosciences. XMIR technology takes advantage of normal cellular processes to package a specific miRNA into exosomes using the XMotif RNA sequence tag. Transfection of XmiR-21-5p to human keratinocytes increased the abundance of miR-21-5p in cells as well as in the exosome isolated from keratinocytes cultured conditioned media (Figure S5A-B). Delivery of TLNP_{κ/si-hnRNPA2B1} to human keratinocyte significantly suppressed hnRNPA2B1 expression (Figure 7A-B). Transfection of XmiR-21-5p to cells treated with either TLNP_{κ/si-control} or TLNP_{κ/si-hnRNPA2B1} showed no significant difference in the number of exosomes released by keratinocytes (Figure 7C). However, quantification of exosomal RNA content and miR-21-5p abundance within the exosome from TLNP_{κ/si-hnRNPA2B1} and XmiR-21-5p treated keratinocytes showed significant reduction suggesting that TLNP_{κ/si-}

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2 hnRNPA2B1 was successful in inhibiting the miRNA packaging within the exosome in keratinocytes
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4 (Figure 7D-F).
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9 **Delivery of keratinocyte-specific lipid nanoparticles encapsulating si-hnRNPA2B1**
10 **compromised quality of wound closure.** Based on observations from *in vitro* studies on the role
11 of Exo_{κ-GFP} in the conversion of macrophage phenotype (Figure S3E), we postulated that *in vivo*
12 delivery of TLNP_{κ/si-hnRNPA2B1} to inhibit miRNA packaging in keratinocytes will compromise the
13 quality of wound healing in mice. Comparable to findings of *in vitro* studies, delivery of TLNP_{κ/si-}
14 hnRNPA2B1 to murine keratinocyte significantly suppressed hnRNPA2B1 expression (Figure 8A-D,
15 Figure S6A). Interestingly, no significant difference in the number of exosomes released at the
16 wound-edge was observed (Figure S6B). Furthermore, wound closure in both TLNP_{κ/si-control} and
17 TLNP_{κ/si-hnRNPA2B1} groups were comparable (Figure 8E, Figure S6C-D). However, at day 10 in
18 mice treated with TLNP_{κ/si-hnRNPA2B1}, persistent accumulation of inflammatory cells was noted in
19 the granulation tissue (Figure 8F). These inflammatory cells were identified to be as ωmφ (Figure
20 8G, Figure S6E). Unlike the scanty ωmφ that exhibit proresolution marker arginase in TLNP_{κ/si-}
21 control group, these ωmφ abundant in TLNP_{κ/si-hnRNPA2B1} treated group expressed proinflammatory
22 marker iNOS even at day 10 post-wounding (Figure 8H-I, Figure S6F-G). Such increased
23 abundance of ωmφ in the granulation tissue of TLNP_{κ/si-hnRNPA2B1} treated group may not be
24 attributed to increased recruitment on the basis of comparable neutrophil or macrophage counts on
25 day 3 post-wounding (Figure S7). These ωmφ, exhibiting proinflammatory phenotype, persisted
26 in the repaired skin even after the wound was closed following TLNP_{κ/si-hnRNPA2B1} treatment
27 (Figure S8). Such presence of proinflammatory ωmφ at the granulation tissue in mice treated with
28 TLNP_{κ/si-hnRNPA2B1} lend credence to the notion that the cues for conversion of ωmφ from
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9 To investigate the significance of abundant proinflammatory $\omega\text{m}\phi$ in the day 10 granulation tissue
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11 of TLNP _{κ /si-hnRNPA2B1} treated mice, we tested the functional property of the re-epithelialized skin
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13 by measuring the barrier function post-closure. The barrier function of the TLNP _{κ /si-hnRNPA2B1} group
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15 was significantly compromised demonstrating that indeed wound closure was impaired (Figure
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17 9A). Restoration of barrier function of the repaired skin is a necessary component of functional
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19 wound healing.^{44, 47-49} The terminally differentiating structural protein loricrin forms 70-80% of
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21 the cornified envelope contributes to the protective barrier function of skin.⁴⁹⁻⁵¹ The abundance of
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23 loricrin in the re-epithelialized skin was significantly compromised following TLNP _{κ /si-hnRNPA2B1}
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25 treatment (Figure 9B). Furthermore, lower expression of other junctional proteins such as ZO-1,
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27 ZO-2, filaggrin and occludins was also observed at day 10 post-wounding in TLNP _{κ /si-hnRNPA2B1}
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29 treated group compared to TLNP _{κ /si-control} group (Figure 9C). Taken together, these observations
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31 explain how functional wound closure is impaired in mice treated with TLNP _{κ /si-hnRNPA2B1}. Thus,
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33 impairment in miRNA packaging in keratinocyte exosomes of the skin impaired resolution of
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35 inflammation and compromised functional wound closure.
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CONCLUSION

In summary, we developed a method of isolating $\text{Exo}_{\text{K-GFP}}$ in tissue for studying keratinocyte-exosome crosstalk during wound healing. This work provides critical insight into the significance of $\text{Exo}_{\text{K-GFP}}$ in the resolution of wound inflammation and determination of functional wound closure. $\text{Exo}_{\text{K-GFP}}$ showed abundance of small bp RNA (<100 bp) in day 5 wound-edge tissue. Exo_{K} -borne miRNA signals enable crosstalk between resident keratinocytes of the skin with visiting $\omega\text{m}\phi$. Glycan ions with high mannose was only detected in wound-edge Exo_{K} . Wound macrophages are known to possess mannose receptors. Mannose-functionalization is commonly used to target nanoparticles for $\omega\text{m}\phi$ uptake. In this work, Exo_{K} were taken up by $\omega\phi$ in the wound-edge tissue. Such uptake caused phenotypic changes in $\omega\text{m}\phi$ consistent with the resolution of inflammation. Blockade of miRNA transfer *via* $\text{Exo}_{\text{K-GFP}}$ to $\omega\text{m}\phi$ by knocking down hnRNPA2B1 caused persistence of proinflammatory $\omega\text{m}\phi$. Thus, exosomal miRNA packaging in skin keratinocytes modify wound inflammation response. Impaired resolution of wound inflammation, caused as above, hindered functional wound healing by compromising restoration of skin barrier function at the site of repair. Findings of this work lay the framework of an emerging paradigm wherein exosome-borne molecular signals drive crosstalk between different cellular compartments in a way that directly determines the fate of wound healing outcomes. Such advancement in our understanding of wound healing unveils heretofore unknown therapeutic targets that may be exploited to design productive wound-care strategies.

METHODS

Cells and cell culture. Immortalized human keratinocytes (HaCaT) were grown in Dulbecco’s low-glucose (1g/L) modified Eagle’s medium (Life Technologies, Gaithersburg, MD) as described previously.⁵² Human dermal microvascular endothelial cells (HMECs) were cultured in MCDB-131 medium supplemented 10 mm l-glutamine, and 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin (Invitrogen), as described previously.⁵³ Human skin fibroblast BJ cells (ATCC® CRL-2522™) were obtained from ATCC and were cultured in Eagle's Minimum Essential Medium, (Catalog No. 30-2003) as per the instruction provided. Mouse keratinocytes (KERA-308) were purchased from Cell line Services (CLS Germany) and were cultured in Dulbecco’s high-glucose (4.5g/L) modified Eagle’s medium (Life Technologies, Gaithersburg, MD). Primary mouse embryonic fibroblasts were purchased from Millipore Sigma (PMEF-HL) and were cultured as per manufacturer’s instruction. For isolation of wound macrophages (ωmφ), circular (8 mm) sterile Polyvinyl alcohol (PVA) sponges were implanted subcutaneously on the backs of 8 to 12 week-old mice.⁵⁴ Sponge-infiltrated wound mφ (CD11b⁺) were obtained from day 3 wound cell infiltrate by magnetic bead-based sorting as previously described.^{55, 56} The isolated cells were cultured in RPMI-1640 media. The cells were maintained in a standard culture incubator with humidified air containing 5% CO₂ supplemented with 10% FBS and 1% antibiotic-antimycotic (AA) (Life Technologies) unless stated otherwise. All experiments involving isolation or uptake of exosomes were performed with exosome depleted FBS.

Isolation of exosome from cell culture media (EV-TRACK ID: EV190103). Cell culture supernatant (using cells cultured with media described above and 10% Gibco Exosome-Depleted FBS (ThermoFisher Scientific)) was centrifuged at 3400g for 15 min and the supernatant was collected. Extracellular vesicles were isolated from the supernatant using differential ultracentrifugation (Beckman Coulter Optima Max-XP Ultracentrifuge, rotor TLA120.2) as

described in figure S1. Pellets were washed by resuspending in PBS and re-pelleting *via* a second round of ultracentrifugation. These pellets were then resuspended in PBS and incubated overnight at 4°C with magnetic CD9, CD63 and CD81 Dynabeads (Invitrogen). The exosomes-attached to the beads were magnetically separated from flow through using a magnetic microcolumns (μ columns from Miltenyi Biotec MACS). The flow through eluent (membrane particles and apoptotic bodies) was kept and re-pelleted for further analysis (2h at 245,000g). For Western blot and flow cytometry, the exosomes were not removed from the magnetic beads. For Nanoparticle Tracking Analysis or SEM imaging, exosomes were eluted from the beads using elution buffer (ExoFlow Exosome Elution Buffer, System Biosciences) as per manufacture's protocol. This method was submitted to EV track.²⁷

Isolation of keratinocyte-derived exosome from murine tissue. EVs and keratinocyte-derived exosomes were isolated from mouse tissue following transfection with Keratin 14 promoter driven plasmids encoding murine CD63, CD9 and CD 81 with GFP reporter “in frame”. The murine skin and wound-edge tissue were collected and homogenized, suspended in PBS and vortexed to release exosomes from tissue. The solution was briefly centrifuged and the supernate collected and centrifuged at 5000g for 15 min followed by 20,000 g for 45 min. The supernatant was incubated overnight at 4°C with GFP-Trap magnetic agarose beads (Chromotek Catalog # gtma-100) (12 μ L GFP-Trap beads per 0.15 g tissue). Exosome isolation from GFP-Trap beads utilized magnets to isolate the beads, enabling the eluent to be removed. The GFP-Trap beads were washed thrice in PBS. Finally, elution of intact beads was accomplished by mixing with glycine (0.2M, pH 3) for 30 seconds, a process that was repeated 5 times. The pH was adjusted to 7.4 using 1.0 M Tris, and EXO_{K-GFP} pellets were collected by ultracentrifugation (2 h at 245,000g). The flow through was next incubated with magnetic pan-CD beads (Miltenyi Biotec Catalog # 130-117-039) for 2 h at

room temperature that were isolated from flow through to recover the non-keratinocytes derived exosome as described above for Invitrogen Dynabeads.

Fluorescence correlation spectroscopy (FCS). Exosome-protein binding was also measured by the change in diffusion and anisotropy of a fluorescently labeled antibody (TSG101-PE) incubated in the presence of exosomes or other extracellular vesicles using a two-channel fluorescence correlation spectroscopy system (Confocor 2, Zeiss) attached to an Axiovert200 M inverted microscope (Zeiss).⁵⁷ The system measures a characteristic diffusion time (τ_D) of a fluorophore as determined by fitting fluorescent decay within a confocal volume to an autocorrelation curve using the Eq. (1), where N is particle concentration and Q is a factor relating to the ellipticity of the confocal volume:

$$G(t) = 1 + \frac{1}{N_{avg}} \left(\frac{1}{1 + \tau / \tau_D} \right) \left(\frac{1}{\sqrt{1 + (1/Q^2)(\tau / \tau_D)}} \right)^{58} \quad (1)$$

Autocorrelation best fit curves identify change in particle diffusion with curves shifted to the left demonstrating faster diffusion (smaller τ_D); exemplary best fit curves are shown, where the curves are fitted using a one-component fit identifying an average characteristic diffusion time for the fluorophores. For fluorophores bound to exosomes, this will provide qualitative evidence of exosomal binding.

Fluorescence anisotropy. Fluorescence anisotropy experiments were conducted using a two-channel fluorescence correlation system where both channels are equipped with crossed analyzers, one perpendicular and one parallel to the emitted laser light. The emitted light divides into two separate beams of equal intensity, which are guided into each of the two APD channels. The anisotropy can be calculated using Eq. (2).

$$r = (I_1 - I_2) / (I_1 + 2I_2) \quad (2)$$

where I_1 and I_2 are the intensities of the parallel and perpendicular channels, respectively.^{57, 59} A correction factor for difference in analyzer sensitivity was not utilized as this factor does not alter significance of change in anisotropy between different conditions. Anisotropy experiments are complementary to autocorrelation analysis because they provide information about the short-range mobility of the tracer molecules, namely the change in rotational diffusion. Autocorrelation curves shown are examples of curves collected with concentration normalized; anisotropy data were collected using 10s runs for each data point.

N-Glycan analysis. Denaturation solution, consisting of 0.1% SDS (Bio-Rad Laboratories, Inc., Hercules, CA) and 0.2% β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO) in 50 mM sodium phosphate buffer (pH 7.5), was added to the exosome sample and incubated at 60 °C for 1 h. After that, nonidet P40 substitute (NP-40, Roche Diagnostics Corp, Indianapolis, IN) was added to encapsulate SDS. The sample was then incubated with Peptide N-Glycosidase F (PNGase F, New England BioLabs, Inc., Ipswich, MA) at 37 °C for 18 h. Cleaved N-glycans were purified through solid-phase extraction on an active charcoal phase (Micro SpinColumns, Harvard Apparatus, Holliston, MA). For methylation,^{60, 61} purified and dried glycans were dissolved in 5 μ L of DMSO (Fisher Chemical, Fair Lawn, NJ), containing 2 M methylamine hydrochloride (Sigma-Aldrich, St. Louis, MO) and 1 M 4-methylmorpholine (Sigma-Aldrich, St. Louis, MO), and 5 μ L of 100 mM (7-Azabenzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP, Sigma-Aldrich, St. Louis, MO). The reaction proceeded at room temperature in the dark for 4 h and was terminated with addition of 240 μ L of 85% acetonitrile (OmniSolv, Billerica, MA). Methylamidated N-glycans were purified through solid-phase extraction on a hydrophilic interaction phase (Amino Micro SpinColumns, Harvard Apparatus, Holliston, MA). To impart a +1 charge for electrophoresis, glycans were labeled with Girard's reagent T (GT; Sigma-Aldrich,

1
2 St. Louis, MO). Briefly, 25 μ L of 0.02 M GT in 10% (v/v) acetic acid was added to purified glycans
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4 and incubated at 55 $^{\circ}$ C for 4 h, and the reaction mixture was dried in a CentriVap concentrator to
5
6 remove excess acetic acid and water.
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9 CE-MS analysis³⁵ of the N-glycans was conducted on a CESI 8000 instrument (SCIEX
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11 Separations, Framingham, MA) with a neutral capillary cartridge (30 μ m i.d. x 90 cm length; Opti-
12
13 MS, B07368, SCIEX Separations) and an Orbitrap Fusion Lumos mass spectrometer (Thermo
14
15 Fisher, Waltham, MA). GT-labeled N-glycans were dissolved in 10 μ L of 0.5% acetic acid (v/v).
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17 Sample was injected into the capillary hydrostatically at 3 psi for 25 s and electrophoretically
18
19 separated with an applied potential of 25 kV and pressure of 3 psi. The capillary was connected to
20
21 the mass spectrometer through an electrospray interface with an applied potential of 1090 V. MS
22
23 data from positive ions ranging from 200 to 2000 m/z were collected. The detected masses were
24
25 searched on ExPASy GlycoMod, and tentative structures were assigned based on previously
26
27 reported structures on ExPASy GlyConnect. Relative intensities of the N-glycans were calculated
28
29 with the Thermo Xcalibur Qual Browser algorithm Genesis where the signal was boxcar averaged
30
31 (7 points) with mass tolerance 30 ppm.
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38 **Flow cytometry.** Exosome markers were assessed using PE anti-TSG101 (1:100, NB200-112PE,
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40 Novus Biologicals), PE anti -flotillin-1 (1:200, ab225165, Abcam) by incubating the antibodies at
41
42 the noted concentrations with the beads for 90 minutes at room temperature. Additional exosome
43
44 markers, anti-Alix (1:100, 92880 Cell Signaling Technology), and anti-HSP90 (1:100, ab59459,
45
46 Abcam) were first incubated with beads for 90 minutes at room temperature followed by magnetic
47
48 separation using PBS to remove excess antibodies and incubation of beads next with the secondary
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50 antibody. Goat Anti-Rabbit IgG Alexa Fluor 488 (1:200, ab150077). PE fluorescence was
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52 determined using PE channel. Alexa Fluor 488 fluorescence was determined using FITC channel.
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For the membrane integrity assay, human keratinocytes were incubated with Propidium iodide (1mg/ml) and the percentage of viable cells was measured in FL2 channel.⁶² Heat killed human keratinocytes were used as positive control. Samples were run on an Accuri C6 flow cytometer (Accuri Cytometers, MI, USA) or LSRFortessa X-20 flow cytometers (BD Biosciences, CA, USA). Data were collected from 5000 – 10,000 events at a flow rate of 250-300 events/s and analyzed using FlowJo software (Tree Star, OR, USA).

Synthesis of DSPE-PEG₂₀₀₀-A5G33. DSPE-PEG₂₀₀₀-A5G33 was synthesized through the conjugation of A5G33⁴⁴ (sequence: ASKAIQVFLLAG, Genscript, NJ, USA) and DSPE-PEG₂₀₀₀-NHS (Nanocs, Boston, MA, USA). Lipid peptide conjugation was done by dissolving 20 mg DSPE-PEG₂₀₀₀-NHS in dimethyl sulfoxide (DMSO) with a solution of A5G33 in DMSO and 10 μ L trimethylamine (TEA). The reaction was performed in an oxygen-free environment (nitrogen purge) at room temperature for 24 hours. The resulting mixture was dialyzed against deionized water using a slide-a-lyzer dialysis cassette (Molecular weight cut-off, MWCO 3500) for 48 h to remove impurities. The final solution was lyophilized and the powder was stored at -20°C for further use. DSPE-PEG₂₀₀₀-A5G33 was characterized using MALDI-TOF mass spectrometry (15 T FT-ICR Bruker Daltonics Inc.).

Preparation of keratinocyte-targeted lipid nanoparticles (TLNP_k). TLNP_k were prepared by using a modified ethanol dilution method as described previously⁴⁴. Briefly, DOTAP/DODMA/DOPC/DSPE-PEG₂₀₀₀-A5G33/Tween80 (20/30/27/3/20, mol/mol) were dissolved in ethanol, and mixed with siRNA in triethylammonium acetate buffer (20 mM, pH 4.5). The mixture was further diluted using PBS (10 mM phosphate, 135 mM NaCl, pH 7.4). The ethanol and free siRNA were removed by dialysis using a slide-a-lyzer dialysis cassette (Molecular weight cut-off, MWCO 20000). DSPE-PEG₂₀₀₀-A5G33 was replaced with DSPE-PEG₂₀₀₀ in the

non-targeted lipid nanoparticles (non-TLNP_k). If lipophilic fluorescence dye DiD were chosen to label the LNPs, 0.2% mol/mol amount of dye was added into the above formulation recipe.

Encapsulation efficiency of TLNP_k. Encapsulation efficiency was performed by the Quant-iT RiboGreen RNA Kit (Invitrogen, Grand Island, NY, USA) as described previously.⁴⁴ The unencapsulated siRNA content and the total siRNA content that was obtained upon lysis of the TLNP_k by 1% Triton were determined according to the manufacturer's instruction using Multi-Mode Microplate Readers (Biotek, Winooski, VT, USA) at 480 nm λ_{ex} and 520 nm λ_{em} . The encapsulation efficiency (EE) of siRNA was calculated with the following equation:

$$\text{EE} = \frac{\text{Total siRNA} - \text{unencapsulated siRNA}}{\text{Total siRNA}} \times 100\%$$

The encapsulation efficiency of siRNA encapsulated keratinocyte targeted lipid nanoparticles (TLNP_{k/si}) was also re-verified using gel retardation assay *via* 1% agarose gel. Electrophoresis was performed at 100 V for 20 min and visualized under a UV transilluminator (Bio-Rad laboratories, CA, USA).

Electron microscopy. The morphology of exosomes and TLNP_{k/si_hnRNPA2B1} was observed by transition electron microscopy (TEM, Japan). Briefly, the particles were ultracentrifuged at 250,000 g at 4 °C, then the pellet was dispersed into deionized water and dropped on a copper grid, stained with NanoVan (vanadium-based negative stain from Nanoprobes.com, Yaphank, New York) and viewed on a Tecnai G2 12 Bio Twin electron microscope (FEI, Hillsboro, OR, USA) at the Electron Microscopy Center at the Indiana University School of Medicine.

The morphology of exosome and other EVs was checked by scanning electron microscopy. Briefly, pellets containing either exosomes or eluent were resuspended in ddH₂O with 0.1% formalin or other buffer and dropped onto clean silica wafers. After drying, samples were desiccated in a

vacuum chamber for at least 12 hours before analysis. Images were obtained after gold sputter coating using a field emission scanning electron microscope (JEOL 7800F, JEOL Japan) at a beam energy of 5 or 10 kV.

Nanoparticle tracking analysis. Mean particle diameter and concentration of extracellular vesicles and TLN_k were analyzed by Nanosight NS300 with a 532 nm laser and sCMOS camera (Malvern, Worcestershire, UK) as previously described.⁴⁴ Briefly, samples were diluted 100: 1 or as needed in fresh milliQ to obtain 5-100 particles/frame. Samples were typically analyzed using 5 runs of 30s collecting 25 frames per second (749 frames per run) with viscosity determined by the temperature and camera level highest available for sample (typically 15 or 14). The syringe pump speed was 60. NTA automatically compensates for flow in the sample so only Brownian motion is used for size determination. For processing results, the detection threshold was typically 5 with automated blur size and max jump distance. Standard 100 nm latex spheres were run at 1000:1 dilution in milliQ to check the instrument performance. Data were analyzed by NTA 3.0 software (Malvern Instruments).

Zeta potential analysis. Surface charge (ζ potential) measurement of TLNP_k was determined by Zetasizer (Nano-Z, Malvern Instruments Ltd., UK). All samples were dispersed in double-distilled water and tested in volume-weighted size distribution mode. Aliquots of TLNP_k containing anti-miR-107 were diluted in PBS with a series of pHs (50mM, from 2-11) to determine the pH dependency of surface charge.

LDH release assay. Cytotoxicity of TLNP_k was analyzed by measuring the lactate dehydrogenase (LDH) leakage in the media using the TOX-7 *in vitro* toxicology kit (Sigma, St. Louis, MO) as previously described.⁶³ Briefly, HaCaT cells were seeded in 12-well plates (0.1×10^6 cells/well), and incubated overnight. The media were changed when the cells were treated with

1
2 TLNP_{κ/si_hnRNPA2B1}. After incubation for the designated time (24 h and 48 h), cells culture media
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4 were collected and centrifuged, then the supernatant was transferred to a 96-well plate and
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6 incubated with the mixture of the assay substrate, enzyme and dye solutions for 20-30 min in dark
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8 at room temperature. The reaction was terminated by adding 1N HCl to each well. LDH
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10 absorbance was measured at 490 nm using the Bio-TEK ELX 808IU micro plate reader (Bio-TEK
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12 INSTRUMENTS, INC, Winooski, VT).
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17 **MTT assay.** Viability of keratinocytes post nanoparticle treatment was measured using a Vybrant
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19 MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific) per the manufacturer's instructions.
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21 At 24 h after treatment, cells were incubated in medium containing 3-(4,5-dimethylthiazol-2-yl)-
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23 2,5-diphenyltetrazolium bromide (MTT) for 4–6 h at 37°C with 5% CO₂. After MTT treatment,
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25 medium was removed, and DMSO was added (10–20 min at 37°C with 5% CO₂) to solubilize
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27 formazan produced as a result of MTT metabolism. DMSO extract from each well (100 μl) was
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29 collected in a 96-well plate, and formazan content was determined by reading absorbance at 540
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31 nm.⁶⁴
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37 **Lipid nanoparticles and exosome uptake assay.** For the cellular uptake of keratinocyte-targeted
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39 lipid nanoparticles studies, mixed culture of three different cells was performed. 1×10^5 cells
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41 (HaCaT: HMEC: BJ1=1:1:3) were seeded in a 12-well plate in mixed media with 10% FBS and
42
43 1% antibiotics and incubated overnight. DiD-loaded TLNP_{κ/si_hnRNPA2B1} and non-
44
45 TLNP_{κ/si_hnRNPA2B1} were prepared and added into the cells, then incubated at 37 °C. After 2 hours,
46
47 cells were washed using PBS and fixed with 4% paraformaldehyde for 15 min at room temperature.
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49 HaCaT cells and HMEC were immunostained by K14 and CD31 respectively. Confocal images
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51 were acquired with a confocal laser-scanning system (CARL ZEISS confocal microscope LSM
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53 888). For live imaging of the uptake of TLNP_κ by Kera 308, Kera cells were seeded in 4-well
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2 tissue culture chambers at a density of 2×10^4 cells and incubated overnight. DiD-loaded
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4 TLNP_{κ_FAM-siRNA} and non-TLNP_{κ_FAM-siRNA} were prepared and incubated with the cells. The
5
6 cellular uptake was visualized for about 95 minutes.
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10 For uptake of exosome studies by wound macrophages, Exo_{κ-GFP} were isolated from murine skin.
11
12 The concentration of the exosome was measured by Nanoparticle tracking analysis. The exosomes
13
14 were stained with DiO and added to the day 3 wound and live cell imaging was performed. For
15
16 blocking the C-type lectin receptors, a cocktail of neutralizing antibodies was used. Murine mincle
17
18 (Mabg-mm1; Sigma), mouse dectin 1 (MAB17561; R&D systems) and mouse SIGNR1 (AF18836;
19
20 R&D systems) were used.
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23
24

25 **Ultrahigh Resolution Fourier Transform Mass Spectrometry.** The accurate mass of the
26
27 samples was determined using mass spectrometry. High-resolution mass spectrometry analyses
28
29 were carried out in The Ohio State University, Campus Chemical Instrument Center's Mass
30
31 Spectrometry and Proteomics Facility (OSU CCIC MSP) by using a 15 T Bruker SolarixR FT-
32
33 ICR instrument (Bruker Daltonics, Billerica, MA).⁶⁵ Matrix-assisted laser desorption/ionization
34
35 (MALDI) was used with the alpha cyano hydroxycinnamic acid (HCCA) matrix in the positive
36
37 ion mode. The matrix was purchased from Bruker Daltonics (Billerica, MA). The third harmonic
38
39 of a Yag/Nd laser was used (351 nm) for MALDI with a 25% laser power. The samples were
40
41 mixed with the saturated solution of the HCCA matrix with a matrix: analyte ratio of around 100:1.
42
43
44
45 The resolution of the FT-ICR instrument was set to 150,000 at around m/z 2,500. The detection
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47 range was m/z 300-13,000 but no ions were observed beyond m/z 5,000. Standard FT-ICR ion
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49 optics parameters were used to maximize ion detection efficiency in the applied m/z range.
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Transfection of XmiR-21 mimics. DharmaFECT™ 1 transfection reagent was employed to transfect HaCaT cells with XmiR-21 (100nM) (Dharmacon) as described previously.⁴⁹ Cells and media were collected 48 h after transfection for further analysis as indicated.

Animals. Male C57BL/6 mice (aged 8-10 weeks) were obtained from Jackson Laboratory. All animal studies were performed in accordance with protocols approved by the Laboratory Animal Resource Center of Indiana University. No statistical methods were used to predetermine the sample size. Power analysis was not necessary for this study. The animals were tagged and grouped randomly using a computer-based algorithm (www.random.org).

Wound models and *in vivo* TLNP_{κ/siRNA} delivery. For wounding, two 6-mm diameter full-thickness excisional wounds were developed on the dorsal skin of mice with a 6-mm disposable biopsy punch and splinted with a silicon sheet to prevent contraction thereby allowing wounds to heal through granulation and re-epithelialization.⁶⁶⁻⁶⁸ TLNP_{κ/si-control} and TLNP_{κ/si-hnRNPA2B1} were administrated into the wound edge by subcutaneous injection. For isolation of exosomes from wound-edge, four 8 mm diameter full-thickness excisional wounds were developed on the dorsal skin of mice with an 8-mm disposable biopsy punch without any stent. During the wounding procedure, mice were anesthetized by low-dose isoflurane (1.5%-2%) inhalation as per standard recommendation. Tissue from the wounds (skin) was snap-frozen and stored at -80°C until harvested for exosome collection described above. Each wound was digitally photographed at the time point indicated. Wound size was calculated by the ImageJ software.²²

The animals were euthanized at the indicated time and wound edges were collected for analyses. For wound-edge harvest, 1–1.5 mm of the tissue from the leading edge of the wounded skin was excised around the entire wound. The tissues were snap-frozen and if used for exosome harvest,

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2 left in -80°C until harvested for exosome collection described above. If used for IHC, tissues were
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4 collected either in 4% paraformaldehyde or in optimal cutting temperature (OCT) compound.
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8 **Tissue nanotransfection 2.0.** *In vivo* TNT was performed as described previously with a
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10 modification in the chip design.³¹ The hollow microneedle array was fabricated on a double side
11
12 polished Silicon wafer using a standard semiconductor process in a cleanroom environment. First,
13
14 the Si wafer was wet oxidized in a furnace at 1150 °C to grow 4 µm thermal oxide on both sides
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16 that served as a hard mask during the deep silicon etching. A 10 µm thick, positive photoresist of
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18 AZ 9260 was spin-coated on one side of the silicon wafer followed by a prebake at 110 °C for 10
19
20 min. A direct laser writing system was used to expose a layout of 25 µm circle arrays followed by
21
22 development in a diluted AZ400K solution to remove the exposed area. The 4 µm oxide was
23
24 removed by a plasma etcher using CHF₃ chemistry. The wafer was then transferred to another
25
26 plasma etching system to perform a deep Si etching called Bosch process, a common
27
28 semiconductor process to achieve a vertical etching profile with a high-aspect ratio. After silicon
29
30 etching of about 350 - 450 µm in depth to form the reservoir arrays, the wafer was flipped for the
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32 next step to etch the hollow microneedle arrays. A donut-shaped pattern was exposed onto the
33
34 resist and the pattern was transferred to the oxide using the same set of steps mentioned above.
35
36 Then, the wafer was etched by the Bosch process until the hollow microneedles are connected to
37
38 the reservoirs so that the cargo or the plasmid DNA fluid can freely flow from the reservoir to the
39
40 hollow microchannel. The SEM images showed the fabricated silicon hollow microneedle array
41
42 (Fig. 1C) that has a length of 170 µm, an outer diameter of 50 µm and a hollow diameter of 4 µm.
43
44 When an electric pulse was applied between the TNT chip and the tissue, the negatively charged
45
46 plasma DNA will travel from the reservoir to nearby target cells by electrophoresis and enter them
47
48 by electroporation. To test the TNT2.0 delivery efficiency, FAM-DNA (5'/56-
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50 FAM/TACCGCTGCGACCCTCT-3') was used in murine skin.
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Trans-epidermal water loss. TEWL serves as a reliable index to evaluate the skin barrier function *in vivo* 23-25. TEWL was measured from the skin and wounds using DermaLab TEWL Probe (cyberDERM, Broomall, PA). The data were expressed in $\text{g}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$.

RNA extraction and quantitative real-time PCR. RNA from cells or exosome was extracted using miRVana miRNA isolation kit (Ambion) according to the manufacture's protocol.^{21, 69} For determination of miR expression, specific TaqMan assays for miRs and the TaqMan miRNA reverse transcription kit were used, followed by real time PCR using the Universal PCR Master Mix (Applied Biosystems, Foster City, CA). mRNA was quantified by real-time or quantitative (Q) PCR assay using the double-stranded DNA binding dye SYBR Green-I.^{21, 69}

High-resolution automated electrophoresis of RNA. The RNA isolated from Exo_K-GFP was analyzed in Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California).⁷⁰

Western blots. Western blot was performed using antibodies against human hnRNPA2B1 (Sigma-Aldrich; SAB1403931, 1:500), mouse hnRNPA2B1 (Sigma-Aldrich; HPA001666, 1:200), TSG101 (Abcam; ab125011, 1:1000), Alix (Novus Biologicals; JM 85-31, 1:1000), HSP90 (Abcam; ab59459, 1:1000), Flotillin 1 (Abcam; ab133497, 1:200), GM130 (Abcam; ab52649, 1:1000), Prohibitin (Abcam; ab28172, 1:200). Signal was visualized using corresponding HRP-conjugated secondary antibody (Amersham, 1:3,000) and ECL Plus™ Western Blotting Detection Reagents (Amersham). β -actin (Sigma-Aldrich; A5441, 1: 2000) served as loading control.^{21, 22}

Immunohistochemistry (IHC) and microscopy. Immunohistochemistry was performed as described previously.⁷¹ Immunostainings of F4/80 (Bio-Rad , MCA497R; 1:200), K14 (Covance, PRB-155P-100; 1:400), GFP (Abcam, ab13970; 1:500), MPO (Abcam, ab9535; 1:50), Arginase (Abcam; 203490, 1:100), iNOS (Abcam; ab115819, 1:100), hnRNPA2B1(Sigma-Aldrich;

1
2 HPA001666, 1:50), Occludin (Invitrogen, 711500; 1:200), ZO-1 (Invitrogen, 617300; 1:200),
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4 ZO-2 (Invitrogen, 389100; 1:200), Loricrin (Biolegend, PRB-145P; 1:400) , Filaggrin (Covance,
5
6 PRB-417P; 1:500) CD31 (BD Pharmingen, 550274; 1:400), Col1A2 (Santa Cruz, sc-393573;
7
8 1:200), CDH5 (Abcam; ab91064, 1:200), were performed on paraffin and cryosections of skin
9
10 sample using specific antibodies as indicated.⁵⁷ The specificity of the antibodies was validated
11
12 using rabbit isotype control (Abcam, ab27478; 1:400). Briefly, the sections blocked with 10%
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14 normal goat serum, and incubated with specific antibodies overnight at 4°C. Signal was visualized
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16 by subsequent incubation with fluorescence-tagged appropriate secondary antibodies (Alexa 488-
17
18 tagged α -rabbit, 1:200; Alexa 568-tagged α -rabbit, 1:200; Alexa 568-tagged α -rat, 1:200, Alexa
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20 488-tagged α -chicken, 1:200) and counter stained with DAPI. Images were captured by
21
22 microscope using super-resolution airyscan laser-scanning confocal system (CARL ZEISS
23
24 confocal microscope LSM 888) and (Axio Scan.Z1, Zeiss, Germany). Quantification of
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26 fluorescent intensity of image was analyzed using Zen software (Zen blue 3.1) and ImageJ
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28 software with colocalization plugin.^{72, 73}
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35 **Statistical analysis.** GraphPad Prism (GraphPad Software) v8.0 was used for statistical analyses.
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37 No statistical methods were used to predetermine the sample size. The $\Delta\Delta C_t$ value was used for
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39 statistical analysis of all RT-qPCR data. Statistical analysis between multiple groups were
40
41 performed using one-way analysis of variance with the *post-hoc* Sidak or Bonferroni multiple
42
43 comparison test. Statistical analysis between two groups were performed using unpaired Student's
44
45 two-sided t tests. $P < 0.05$ was considered statistically significant. Significance levels and exact P
46
47 values were indicated in all relevant figures. Data were assumed to be normally distributed for all
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49 analyses conducted. Data for independent experiments were presented as means \pm SEM unless
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60 otherwise stated.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge

Isolation and characterization of exosomes from human keratinocyte conditioned culture media (S1); identification and isolation of keratinocyte-derived exosome in murine skin (S2); conversion of wound macrophage phenotype following uptake of keratinocyte derived exosome (S3); specificity, uptake, and cytotoxicity of human keratinocyte-targeted lipid nanoparticles (TLNP_κ) (S4); abundance of miR-21-5p in keratinocytes and exosome after transfection of XmiR-21-5p (S5); delivery of TLNP_κ encapsulating si-hnRNPA2B1 did not change hnRNPA2B1 expression in other cell types and did not compromise Exo_κ-GFP abundance or wound closure (S6), did not compromise recruitment of inflammatory cells post-wounding (S7), and results in persistence of inflammatory cells post-wound closure (S8). N-glycan analysis of exosome isolated from skin and wound-edge tissue (Table S1). Live-cell confocal images showing uptake of non-TLNP_κ and TLNP_κ by mouse keratinocytes using LSM 880 (mp4 movies S1-S2). Live-cell confocal images showing uptake of Exo_κ-GFP by mouse wound macrophages with or without inhibition of C-type lectin using LSM 880 (mp4 movies S3-S4).

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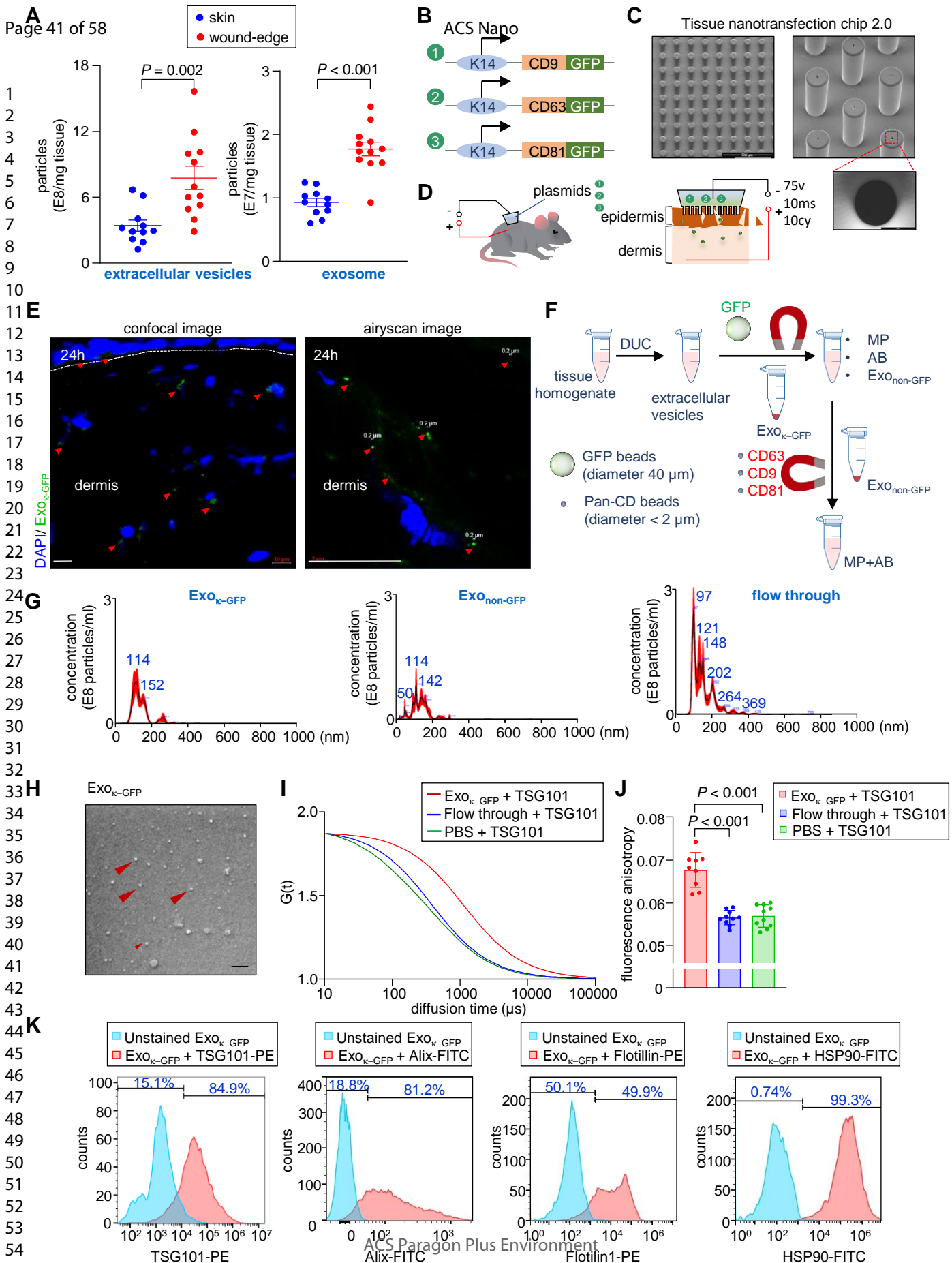


Figure 1

Figure 1. Isolation and characterization of keratinocyte-derived exosomes from murine skin.

(A) Quantification of extracellular vesicles and exosomes from murine skin and day 5 wound-edge tissue of C57BL/6 mice. (n=11, 12) (B) Schematic diagram of keratin 14 (K14) promoter-driven recombinant plasmids encoding CD9, CD63 or CD81 with “in frame” GFP reporter. (C) Representative scanning electron microscopic images of tissue nanotransfection (TNT) chip 2.0. (D) Schematic diagram showing the delivery of the three K14 promoter-driven plasmids *via* TNT in the dorsal murine skin. (E) Confocal microscopic images showing presence of keratinocyte derived exosome (Exo_{K-GFP}) in dermis (left) and super-resolution confocal microscopic images showing GFP labelled exosome (right). The white dash line shows epidermal-dermal junction. Scale, 10µm and 2µm. (F) Schematic diagram showing exosome isolation process from murine skin tissue post-TNT with the three plasmids. MP: membrane particles; AB: apoptotic bodies. (G) Particle size distribution of keratinocyte-derived exosomes (Exo_{K-GFP}), non-keratinocyte-derived exosomes (Exo_{non-GFP}), and flow through (membrane particles and apoptotic bodies) from murine skin. (n=10) (H) Representative Scanning Electron Microscopy (SEM) images of murine keratinocyte-derived exosomes. Scale, 100 nm. (I-J) Binding of TSG-101 PE with the murine keratinocyte-derived exosome was further tested by autocorrelation curves as determined by fluorescence correlation spectroscopy (FCS) (I) and time-resolved fluorescence anisotropy (J). (n=10) (K) Flow cytometric analysis of murine skin keratinocyte-derived exosome on GFP-trap magnetic beads showing binding of TSG-101 PE, Alix-FITC, Flotillin 1-PE and HSP90-FITC antibodies. The histograms demonstrate the shift in fluorescence after binding of murine Exo_{K-GFP} on the GFP-trap magnetic beads with the respective antibody. All data were shown as mean ± SEM. Data in A were analyzed by two-tailed unpaired Student’s t-test. Data in J were analyzed by one-way ANOVA with the *post-hoc* Bonferroni’s multiple comparison test.

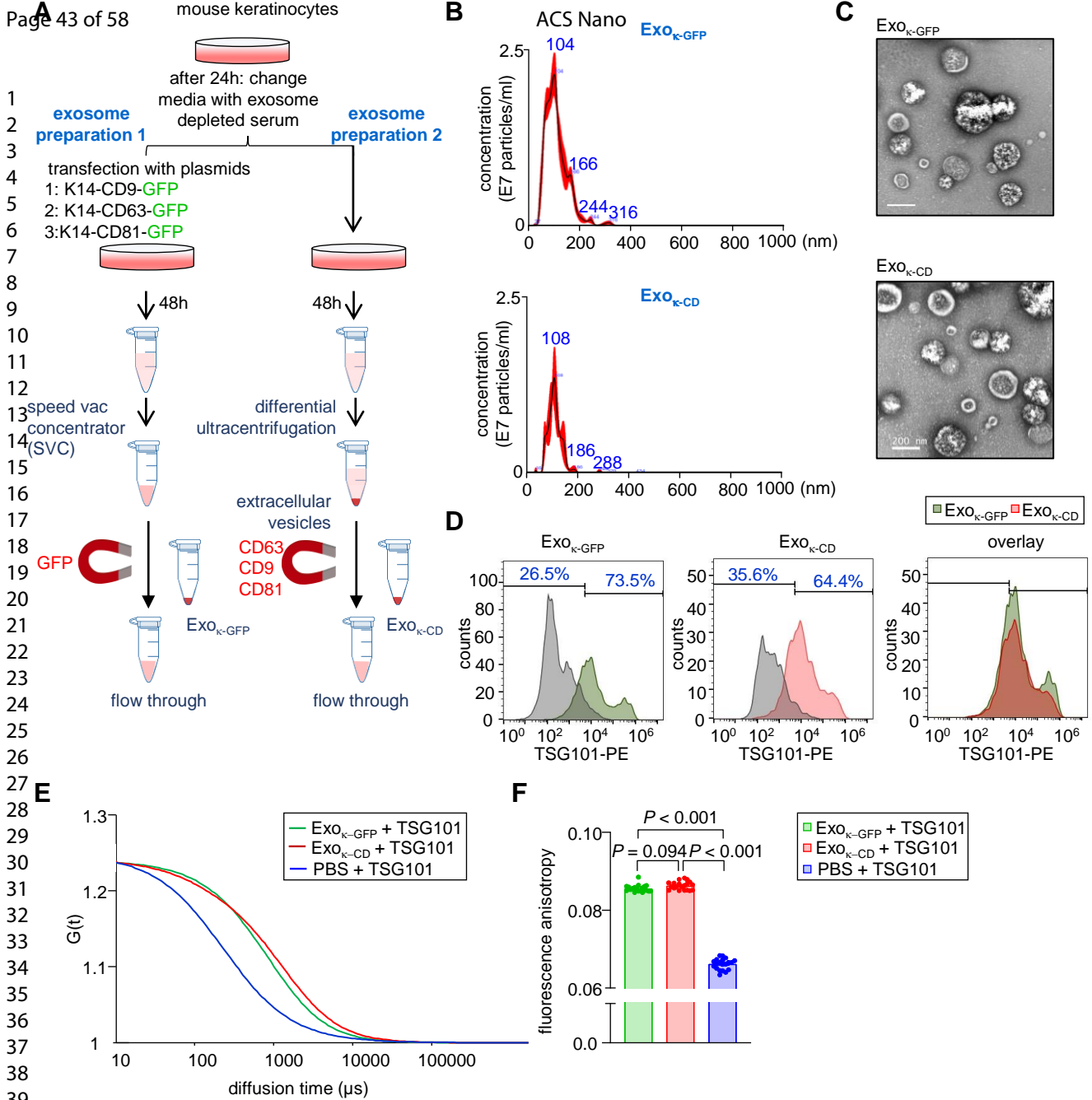


Figure 2

Figure 2: Comparative analysis of keratinocyte-derived exosome with exosome isolated from cell culture conditioned media. (A) Schematic diagram showing exosome isolation process from murine keratinocyte (Kera308) cultured media with and without transfection with keratin 14 promoter-driven recombinant plasmids encoding CD9, CD63 or CD81 with “in frame” GFP reporter. (B) The particle size distribution of exosomes and eluent (membrane particles and apoptotic bodies) from murine keratinocyte cultured media. (n=6) (C) Representative Transmission Electron Microscopy (TEM) images of exosomes isolated using GFP beads and CD-beads. Scale, 200 nm (D) Flow cytometric analysis of murine keratinocyte-derived exosome on CD magnetic beads (CD63, CD9 and CD81) and GFP-beads showing binding of TSG-101 PE antibody. (E-F) Binding of TSG-101 PE with the murine keratinocyte-derived exosome was further tested by autocorrelation curves as determined by fluorescence correlation spectroscopy (FCS) (E) and time-resolved fluorescence anisotropy (F) (n=20). All data were shown as mean \pm SEM. Data in F were analyzed by one-way ANOVA with the *post-hoc* Bonferroni’s multiple comparison test.

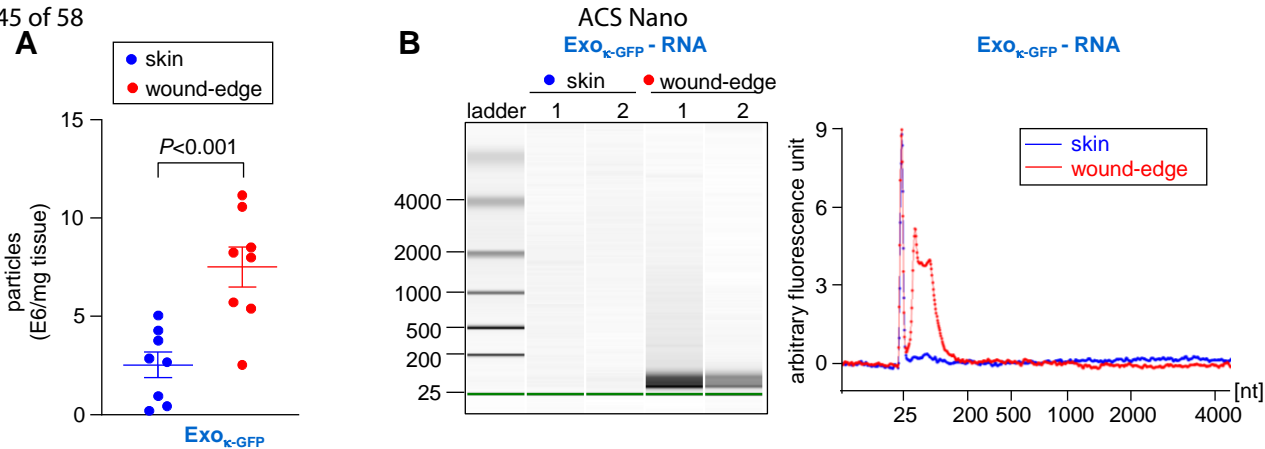


Figure 3

Figure 3. Characterization of keratinocyte-derived exosome from murine skin and wound-edge tissue. (A) Murine dorsal skin was transfected with keratin 14 promoter-driven recombinant plasmids encoding CD9, CD63 or CD81 with “in frame” GFP reporter *via* TNT. The keratinocyte-derived exosomes were isolated from skin and day 5 wound-edge tissue using GFP magnetic trap and quantified using NTA. Data shown as mean \pm SEM and were analyzed by two-tailed unpaired Student’s t-test. (n=8) (B) High-resolution automated electrophoresis of RNA isolated from skin and wound-edge Exo_{K-GFP}. RNA in wound-edge Exo_{K-GFP} was significantly abundant compared to that of skin Exo_{K-GFP}.

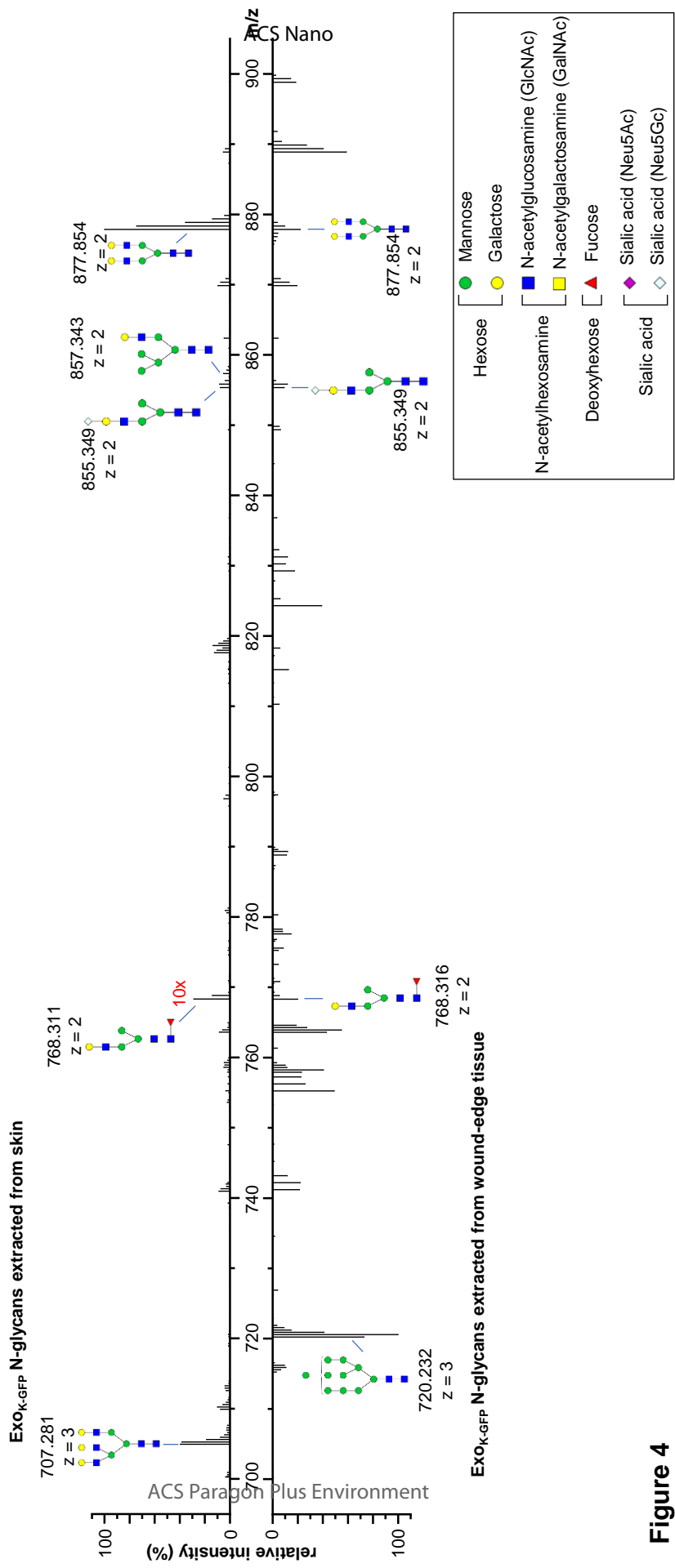


Figure 4

Figure 4. Comparative glycomics of the Exo_K-GFP isolated from skin and wound-edge tissue.
Representative m/z ratios, relative intensities, and proposed structures of N-glycans released from skin and wound-edge tissue derived Exo_K-GFP, methylamidated, labeled with Girard's reagent T, and analyzed by CE-MS. Data analysis determined that 19 and 7 structures were found exclusive to skin and day 5 wound-edge tissue, respectively.

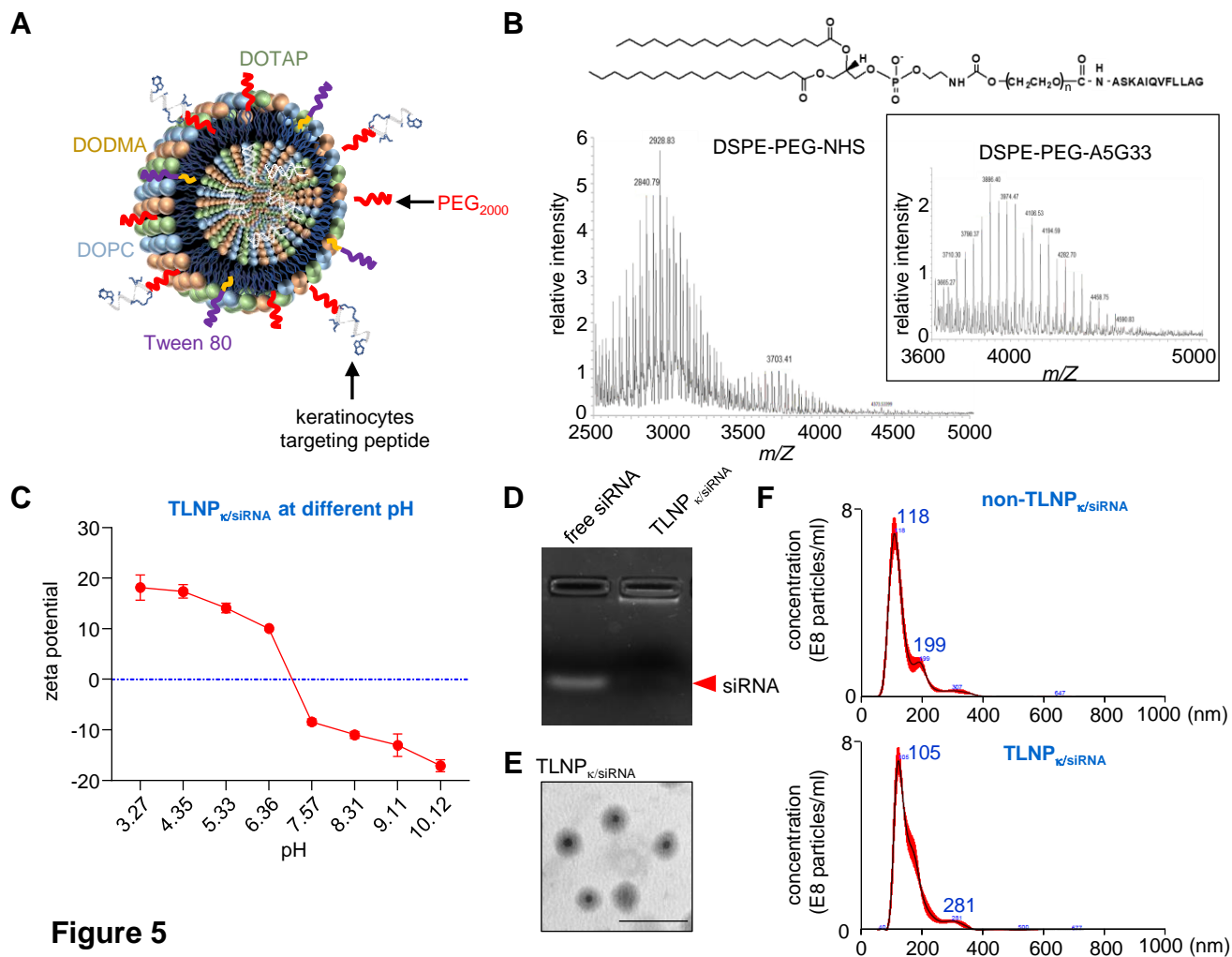


Figure 5

Figure 5: Design and synthesis of keratinocyte-targeted lipid nanoparticles (TLNP_κ) to inhibit miRNA packaging within exosome. (A) Schematic representation of the keratinocyte-targeted lipid nanoparticles TLNP_κ. (B) Mass spectrometric analysis of DSPE-PEG₂₀₀₀-A5G33 and DSPE-PEG-NHS using MALDI-TOF. (C) Zeta potentials of TLNP_κ at different pH. (D) Gel retardation assay of TLNP_κ showing encapsulation efficiency of TLNP_κ. (E) Representative Transmission Electron Microscopy (TEM) image of TLNP_κ. Scale, 500 nm. (F) Representative nanoparticle tracking analysis (NanoSight) showing particle size distribution and concentration of non-TLNP_κ and TLNP_κ.

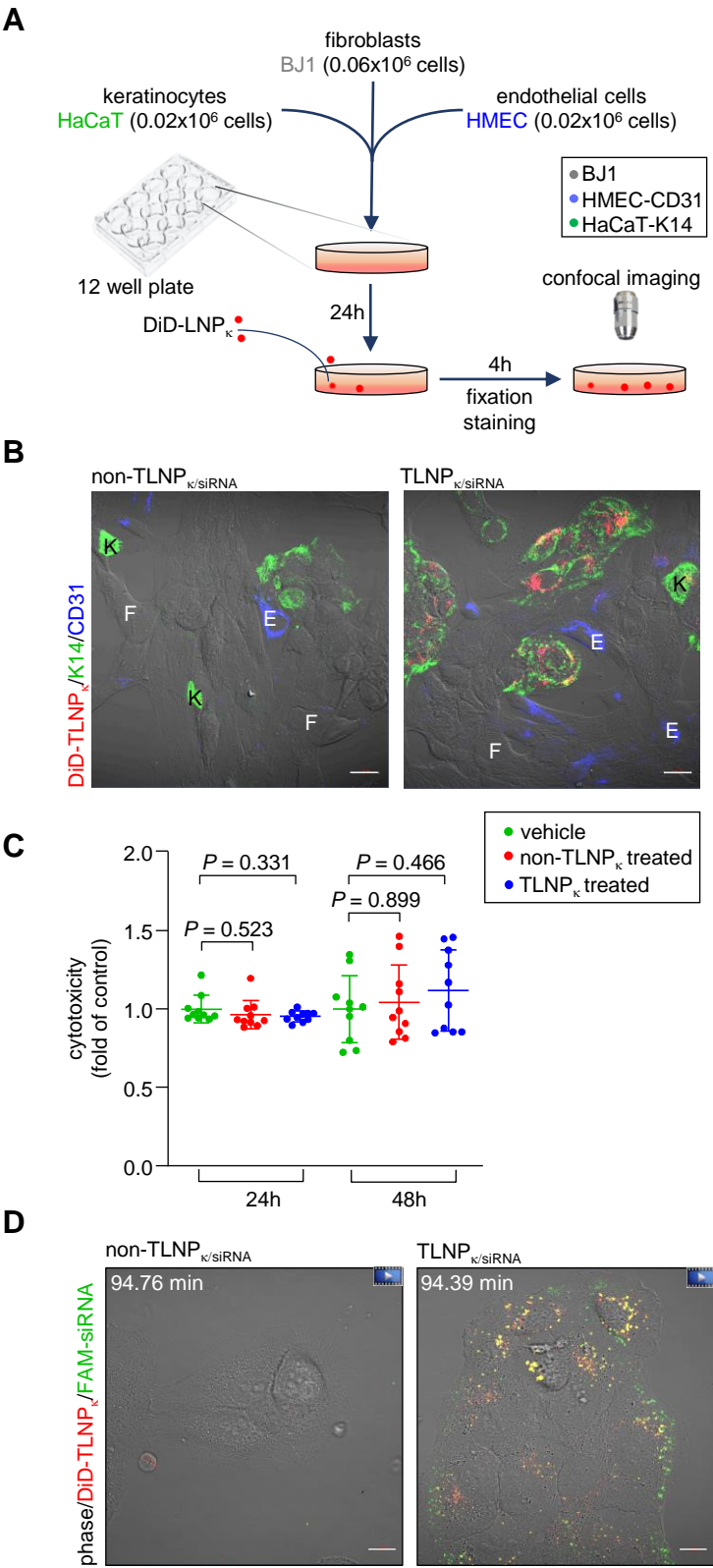

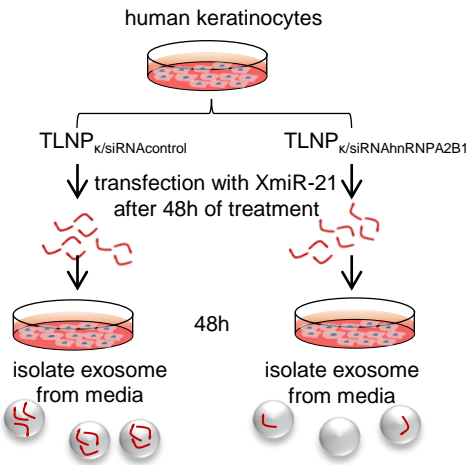


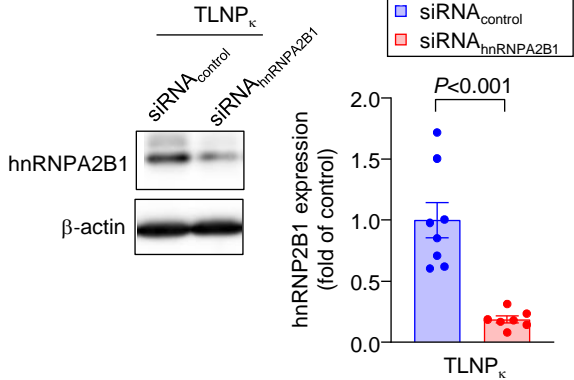
Figure 6

Figure 6: Specificity, uptake and cytotoxicity of keratinocyte-targeted lipid nanoparticles (TLNP_k). (A) Schematic diagram showing experimental design to test the specificity of TLNP_k in mixed culture. (B) Confocal microscopic images showing selective uptake of TLNP_k by human keratinocytes at 4h compared with non-TLNP_k in mixed culture cells (HaCaT: HMEC: BJ-1=1:1:3). K, keratinocyte; F, fibroblasts; E, endothelial cells. Scale; 20 μ m. (C) *In vitro* LDH assay of TLNP_k. (n=10) (D) Live-cell confocal images showing rapid uptake of TLNP_k by mouse keratinocytes compared to non-TLNP_k. Scale, 10 μ m.  Indicate movies in the supplement. Data in C were shown as mean \pm SEM and were analyzed by one-way ANOVA with the *post-hoc* Sidak's multiple comparison test.

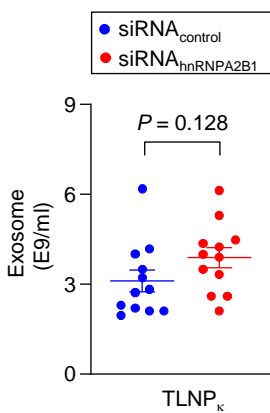
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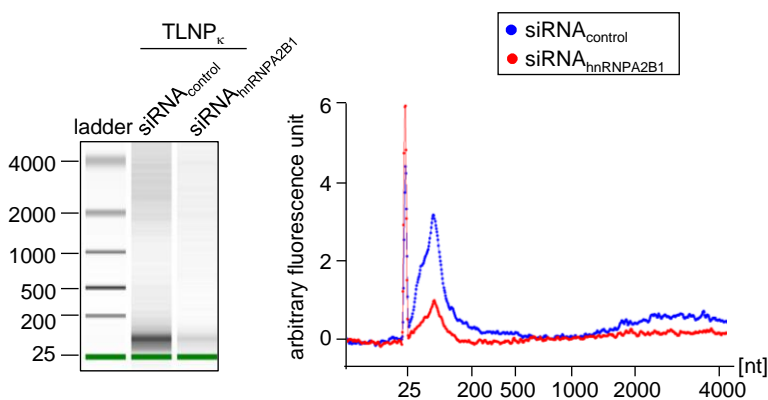
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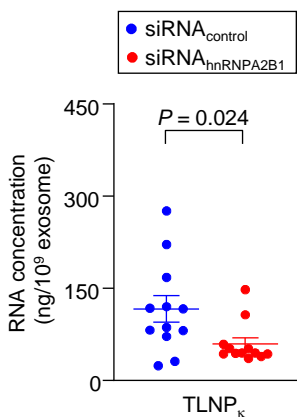
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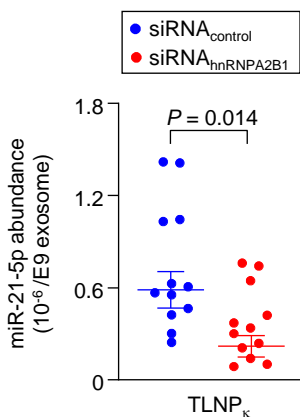


Figure 7

Figure 7: TLNP_k/si-hnRNPA2B1 inhibits packaging of miRNA within the exosome in keratinocyte.

(A) Schematic diagram showing experimental design to test the efficacy of TLNP_k/si-hnRNPA2B1 in inhibiting miRNA packaging within the exosome in keratinocyte. (B) Western blot analysis of hnRNPA2B1 in human keratinocytes 72 h after treatment with TLNP_k encapsulating either si-control or si-hnRNPA2B1. Quantification of hnRNPA2B1 expression from immunoblots. (n=8,7) (C) The exosomes isolated from keratinocyte conditioned media 48h after transfection and Nanoparticle Tracking Analysis was done. The exosome concentration in the conditioned media 48 h after treatment with TLNP_k encapsulating either si-control or si-hnRNPA2B1 were plotted graphically. (n=12) (D) High-resolution automated electrophoresis of RNA isolated from exosomes in the conditioned media 48 h after treatment with TLNP_k encapsulating either si-control or si-hnRNPA2B1 were plotted graphically. (E) The RNA concentration per 10⁹ exosomes in the conditioned media 48 h after treatment with TLNP_k encapsulating either si-control or si-hnRNPA2B1 were plotted graphically. (n=12) (F) The abundance of miR-21-5p in exosome isolated from the conditioned media 48 h after treatment with TLNP_k encapsulating either si-control or si-hnRNPA2B1 were plotted graphically. (n=12). Data in B, C, E and F were shown as mean ± SEM and were analyzed by two-tailed unpaired Student's t-test.

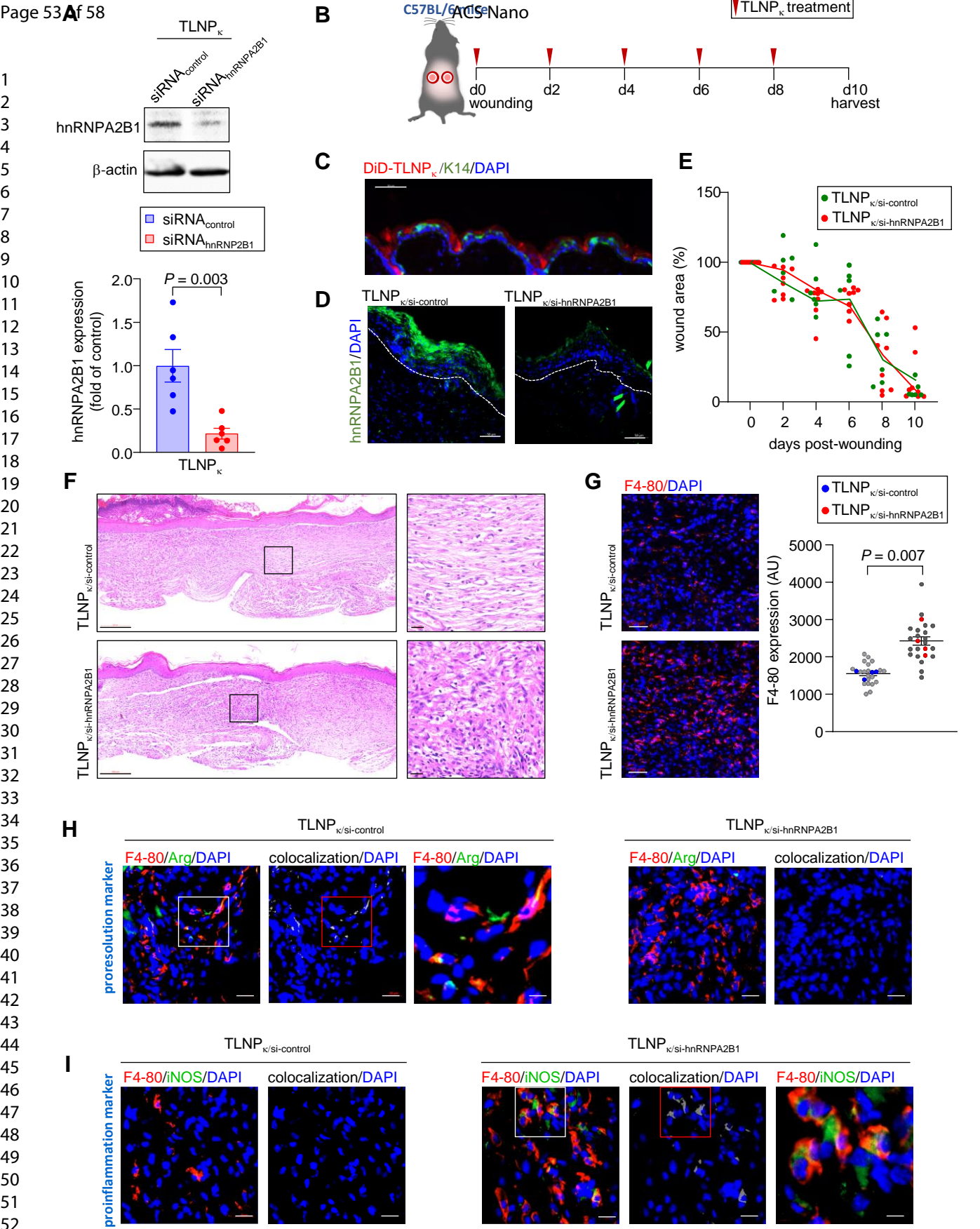


Figure 8

Figure 8: Delivery of keratinocyte-targeted lipid nanoparticles encapsulating si-hnRNPA2B1 compromised the quality of wound closure. (A) Western blot analysis of hnRNPA2B1 in murine keratinocytes 48 h after treatment with TLNP_k encapsulating either si-control or si-hnRNPA2B1. β -actin was used as loading control. Quantification of hnRNPA2B1 expression from immunoblots. (n=6) (B) Schematic diagram showing excisional wounding (6mm stented wound), application of TLNP_k encapsulating either si-control or si-hnRNPA2B1 and tissue harvesting time points in C57BL/6 (wild type) mice. (C) Confocal microscopic image showing localization of DID-labeled TLNP_k (red) in the epidermis (green) at 24h after post-treatment with DID-labeled TLNP_k by subcutaneous injection. Scale, 50 μ m. (D) Representative coimmunofluorescence images showing hnRNPA2B1 (green) and DAPI counterstaining in C57BL/6 mice at day 6 post wounding. White dashed lines indicate the dermal-epidermal junction. Scale, 50 μ m. (E) Quantification of excisional stented punch wounds (6mm) at different days by digital planimetry following delivery of TLNP_k encapsulating either si-control or si-hnRNPA2B1. (n=6,8) (F) Representative Hematoxylin and Eosin (H&E) staining of day 10 murine wound tissue treated with TLNP_k encapsulating either si-control or si-hnRNPA2B1. Scale, 200 μ m (for mosaic images) and 20 μ m (for inset images). (G) Representative coimmunofluorescence staining of F4-80 (red) with DAPI counterstaining in wound-edge tissue at day 10 post-wounding in C57BL/6 mice treated with either scramble or si-hnRNPA2B1 encapsulated keratinocyte targeted lipid nanoparticles. Scale, 50 μ m. Quantification of F4-80 intensity in wound-edge tissue at day 10 post-wounding. Each dot corresponds to one quantified ROI, except the blue and red dots, which correspond to the mean of each mouse. At least 5 ROI per mouse. (n = 4) (H) Representative coimmunofluorescence staining of F4-80 (red) and Arginase (green; proresolution macrophage marker) with DAPI counterstaining in day 10 wound-edge tissue of C57BL/6 mice treated with TLNP_k encapsulating either si-control or si-hnRNPA2B1. The colocalization of red and green are shown as white dots. Scale, 20 μ m. (I) Representative coimmunofluorescence staining of F4-80 (red) and iNOS (green; proinflammatory

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2 macrophage marker) with DAPI counterstaining in day 10 wound-edge tissue of C57BL/6 mice
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4 treated with TLNP_κ encapsulating either si-control or si-hnRNPA2B1. The colocalization of red
5
6 and green are shown as white dots. Scale, 20μm. Data in **A**, **G** and **E** were shown as mean ± SEM
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9 and were analyzed by two-tailed unpaired Student's t-test.
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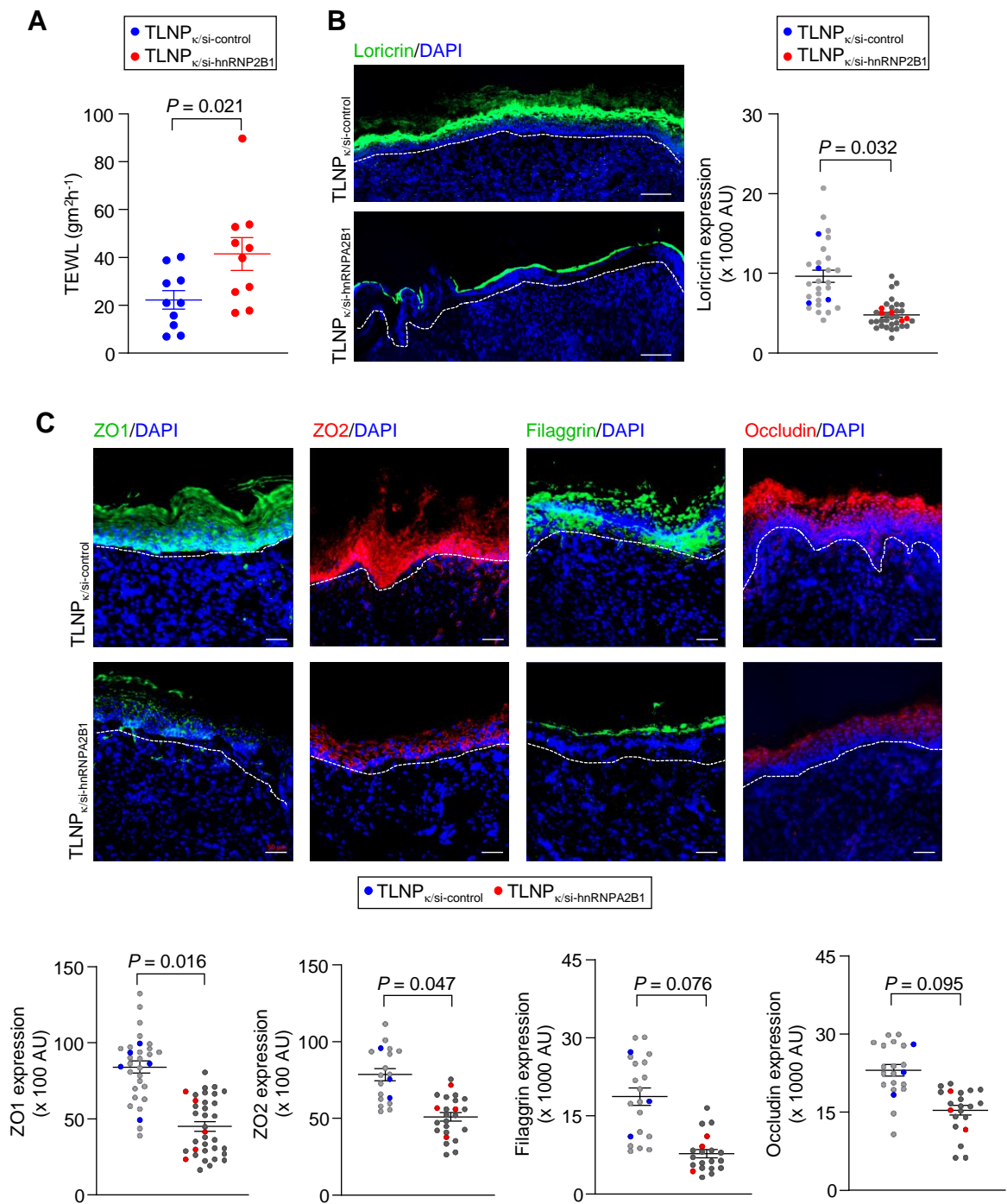
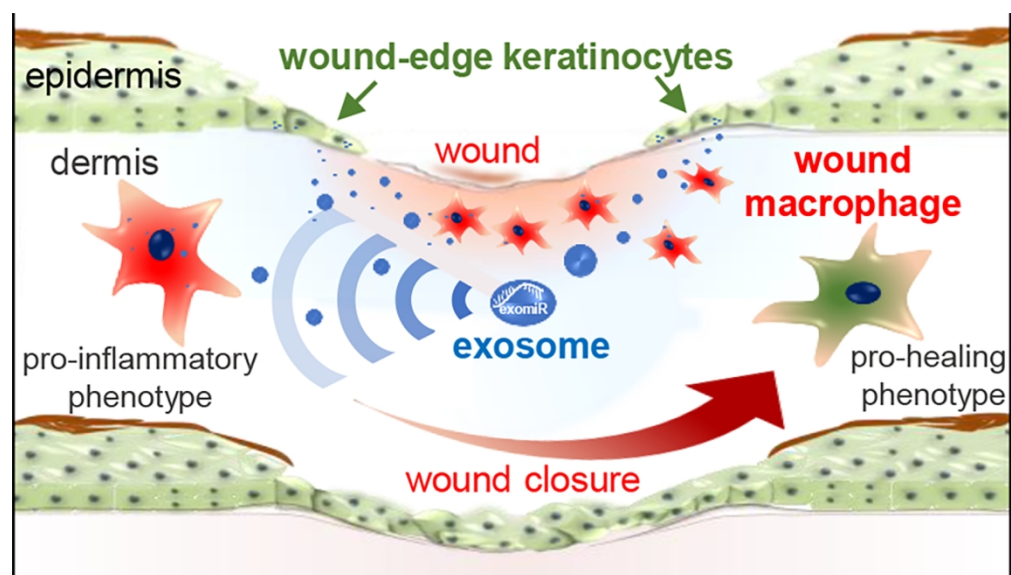


Figure 9: Delivery of keratinocyte-targeted lipid nanoparticles encapsulating si-hnRNPA2B1 compromised the quality of wound closure. (A) Transepidermal water loss (TEWL) after C57BL/6 mice treated with TLNP_κ encapsulating either si-control or si-hnRNPA2B1. (n=10) (B) Representative coimmunofluorescence staining of loricrin with DAPI counterstaining in wound-edge tissue at day 10 post-wounding in C57BL/6 mice treated with either scramble or si-hnRNPA2B1 encapsulated keratinocyte targeted lipid nanoparticles. Scale, 200μm. Quantification of loricrin intensity in wound-edge tissue at day 10 post-wounding. Each dot corresponds to one quantified ROI, except the blue and red dots, which correspond to the mean of each mouse. At least 5 ROI per mouse. (n = 4) (C) TLNP_κ/si-hnRNPA2B1 treatment compromised the expression of ZO-1 (green), ZO-2 (red), filaggrin (green) and occludin (red) in murine skin at day 10. Sections were counterstained with DAPI (blue). Dermal-epidermal junction is indicated by a dashed white line. Scale bars, 50 μm. The abundance of junctional proteins was quantified. Each dot corresponds to one quantified ROI, except the blue and red dots, which correspond to the mean of each mouse. At least 5 ROI per mouse. (n = 3, 4). Data expressed as mean ± SEM and were analyzed by two-tailed unpaired Student's t-test.



Keratinocyte-macrophage crosstalk via exosome